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Influence of pentoxifylline, A-802710, propentofylline and A-802715 (Hoechst) on the expression of cell cycle blocks and S-phase content after irradiation damage

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Abstract

The toxicity of the five methylxanthine derivatives, caffeine, pentoxifylline, A802710, propentofylline and A802715, was determined against the two human melanoma lines, Be11 and MeWo, and against the two human squamous cell carcinoma lines, 4197 and 4451, by vital dye staining assay. Pentoxifylline and A802710 emerge as the least toxic showing TD_{50} (toxic dose of 50%) levels of 3.0–4.0 mM. Propentofylline and caffeine take an intermediate position. A802715 has a TD_{50} of 0.9–1.1 mM and is the most toxic. Subtoxic concentrations ($< TD_{50}$) added after irradiation at maximum expression of the G2/M block show that pentoxifylline and A802710 effectively abrogate the G2/M block, whereas A802715 and propentofylline prolong the G2/M block or remain ineffective depending on the p53 status of the cell line. In p53 wt cells BrdU incorporations show that the irradiation-induced suppression of S-phase entry is marginally enhanced by pentoxifylline but strongly enhanced by propentofylline and A802715. This effect was not seen in p53 mutant cells. Since propentofylline and A802715 prolong the G2/M block and effectively suppress BrdU incorporation these two drugs emerge as antagonists to pentoxifylline, caffeine and A802710. Common structural features of propentofylline and A802715 are a propyl substituent at the N7 position in contrast to pentoxifylline, caffeine and A802710 where the N7 substituent is a methyl group. The results document the effectiveness of four methylxanthines in influencing cell regulation and damage response in human tumor cells.

Keywords: Methylxanthine; G2/M block abrogation; S-phase progression

1. Introduction

Subtoxic doses of pentoxifylline administered to cells after irradiation at maximum expression of the G2 block shortens the recovery period and cells re-enter the cell cycle ahead of time [1–4]. This abrogating effect initiated by pentoxifylline is not measurable when the drug is added immediately before or after irradiation, although improvements of the radiotoxicity have been documented under these conditions [5–8]. In p53 wild type (wt) cells where p53 and its target genes are intact and active, irradiation induces a G1 as well as a G2 block [5]. In p53 mutant cells only a G2 block is induced. The application of pentoxifylline to irradiated p53 mutant and wt cells results in G2 blocked cells re-entering mitosis prematurely, before completing repair of the damaged genome [7–12]. No measurable abrogating effect of

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pentoxifylline is seen on the G1 cell cycle block in wt cells. It is thought that this shortening of the G2 block eliminates a component of the cellular defence and enhances the effectiveness of genotoxic agents [1,2,12–15]. The combination of G2 block abrogators with cytostatic drugs indeed has lead to improvements of cell kill and it is now generally agreed that defective checkpoint controls and the absence of a G1 block in p53 mutants is a situation which may be exploited clinically [2,3,7,8,16,17]. It is not resolved, however, whether cell cycle checkpoint deficiencies apparent in p53 mutant cells provide a definitive advantage for therapeutic intervention. Many checkpoint-activating agents like etoposide, *cis*-Pt or irradiation are not uniformly successful in controlling p53 mutant tumors and it is accepted that checkpoint deficiency alone cannot predict sensitivity to genotoxins. In vitro experiments with p53 and p21 homozygous mutants, on the other hand, suggest that a checkpoint-based strategy may be feasible [18–22]. In this scenario it is clear that G2 block abrogating drugs deserve attention. We have examined the methylxanthine derivatives pentoxifylline, A802710, propentofylline and A802715 for their influence on G2 blocked cells, the G2 block recovery time and entry into S-phase, using pentoxifylline as standard. Experiments were performed on pairs of human melanoma and squamous cell carcinoma lines, where the Be11 and 4197 lines are p53 wt, and the 4451 and MeWo lines carry a G to A mutation in exon 7 codon 258, as described elsewhere [17]. Using a drug dose level below the TD_{50} (toxic dose of 50%) accommodated toxicity differences between drugs and cell lines. The results serve to further evaluate the conditions required for targeted cell deregulation and the possibility of enhancing radiotoxicity by cytotoxic drugs.

2. Materials and methods

The cell lines Be11 (p53 wildtype (wt)), MeWo (p53 mutant) human melanoma cells, 4197 (p53 wt), and 4451 (p53 mutant) human squamous carcinoma were kindly provided by F. Zoelzer and C. Streffer, University of Essen. Cells were grown in modified Eagle's medium with 20% bovine calf serum supplemented with 2 ml/l 1-glutamine, penicillin (100 μg/ml) and streptomycin (100 μg/ml) in 75 cm^2 cell culture flasks (Nunc) under standard conditions and maintained at 37°C with 5% CO_2.

A802715 was kindly supplied by Professor M. Schönharting (Hoechst Marion Roussell, HMR), propentofylline and A802710 was provided by Dr. K. Rudolphi (HMR) and pentoxifylline was obtained from Noristan S.A.

2.1. Determination of the maximum expression of the G2/M block

Irradiation was performed on asynchronous populations of the cell lines with a dose of 7 Gy ^{60}Co γ-irradiation in 25 cm^2 cell culture flasks. Cells were incubated for 4–30 h and harvested according to Ormerod [31] for flow cytometric analysis in two-hourly intervals by trypsinization, centrifugation and fixation in 70% ethanol for at least 30 min at −20°C. Assessment of the DNA was done by flow cytometry. The cells were stained with 10 μg/ml propidium iodide solution (PI, Sigma) to which 100 μg/ml RNase (Boehringer Mannheim) was added. Samples were incubated at 37°C for 30 min and stored at 4°C under light protection.

2.1.1. S phase content

The staining of cells for flow cytometric analysis of DNA content and BrdU incorporation was as described by Wilson [32]. Briefly, at each time point, cells in the exponential growth phase were pulse-labelled for 15 min at 37°C with BrdU. Adding 100 μl of a 1 mM stock solution directly to 10 ml culture medium gave a final BrdU concentration of 10 μM. The BrdU-labelled cells ($\pm 2\times10^6$ cells fixed in 70% ethanol) were denaturated and nuclei released with the HCl/pepsin method before antibody staining. For immunochemical detection of BrdU the cells were resuspended in 0.5 ml phosphate-buffered saline (PBS) containing 0.5% Tween 20, 0.5% normal goat serum and 25 μl mouse anti-BrdU (Dako, M0744). The second step included a cell resuspension in 0.5 ml of PBS containing 0.5% Tween 20, 0.5% normal goat serum and 25 μl anti-mouse FITC-conjugated antibody (Sigma, F-2012). Nuclei collected by centrifugation were counterstained with 10 μg/ml PI at room temperature. Flow cytometric analysis was performed within 3 h after completion of stain-

ing. The % S-phase was measured in a dot plot of FL-2 (red fluorescence or total DNA content by a 600 nm bandpass filter) against FL-1 (green fluorescence or BrdU content by a 544 nm bandpass filter). The BrdU positive part of this plot was used to calculate % S-phase cells sampled at different time points. All measurements were repeated three times.

2.1.2. Determination of DNA content by flow cytometry

DNA analysis was performed using a FACScan flow cytometer (Becton Dickinson) emitting a 488 nm beam. Red fluorescence from PI emission was collected as a linear signal through a 600 nm bandpass filter and recorded as a measure of total DNA content. Processing the red fluorescence into height, area and width (doublet discrimination mode) eliminated cell doublets. Data were collected in list mode and 10 000 events were recorded per sample and displayed as a frequency distribution histogram. Estimates of the percentages of cells in the different periods of postirradiation incubation with marker statistics (LYSIS II software; Becton Dickinson) revealed the time at which the G2/M block was maximally expressed. These times were used for each cell line as a starting point at which the methylxanthine drugs were added. Cell debris, nuclei doublets and triplets were excluded by gating.

2.2. Induction of mitotic block with nocodazole

Nocodazole was dissolved in warm sterile DMSO at a concentration of 1 mg/ml and diluted in DMSO to a stock concentration of 0.1 mg/ml. The working concentration in the culture medium was 0.4 μg/ml. Pentoxifylline was added after 14–16 h when the nocodazole-induced M block had reached a maximum.

2.3. Toxicity studies

The cytotoxicity of the drugs was measured using the crystal violet assay. Cells were seeded at a density of 5000–10 000 cells/well in a 24-well multiplate and grown for 24 h. Cells were cultivated with the drug for 24 h. Four days after change of medium the cells were fixed in buffered formalin (pH 7.2), washed, viable cells stained with crystal violet, and the extracted dye read spectrophotometrically at 590 nm as described [38]. The drug concentration at which the cell survival was reduced to 50% (TD_{50}) was then determined. All measurements were done in triplicate and each experiment was repeated three times.

3. Results

The drugs employed in these studies were caffeine (1,3,7 trimethylxanthine), pentoxifylline (1-(5-oxohexyl)-3,7-dimethylxanthine), A802710 (1-(5-hydroxyhexyl)-3,7-dimethylxanthine), propentofylline (3-methyl-1-(5-oxohexyl)-7-propylxanthine) and A802715 (3-methyl-1-(5 hydroxy, 5 methylhexyl)-7-propylxanthine) (Fig. 1).

Toxicity studies using four representative human tumor cell lines showed that A802715 has a TD_{50} of 0.9–1.1 mM and is by far the most toxic drug. Pentoxifylline and A802710 have a TD_{50} of 3.0–6.0 mM and emerge as the least toxic. Propentofylline and caffeine take intermediate positions with TD_{50}'s of 1.3–2.0 and 2.2–4.0 mM, respectively (Table 1). The effectiveness of the drugs as growth inhibitors was established by the crystal violet vital dye-staining assay (see Section 2) and served to establish the concentration levels at which the drug was beginning to show a growth inhibiting effect. These concentrations (2 mM for pentoxifylline and A802710, and 1 mM for propentofylline and A802715) were then used to

Table 1
Influence of methylxanthine drugs on cell viability as measured by TD_{50} dose (mM) using crystal violet dye staining assay

Cell line	A802715	Propentofylline	Caffeine	Pentoxifylline	A802710
MeWo	1.06	1.3	2.24	3.17	4.3
Be11	0.86	2.0	2.42	6.0	6.0
4451	1.1	2.0	3.22	4.1	3.3
4197	1.07	1.7	3.92	5.6	4.2

test the efficacy of four methylxanthine derivatives in abrogating cell cycle blocks induced by irradiation.

3.1. Drug effects

The effect of irradiation on p53 wt cells is a marginal decline and then blockage of the G1 population and an increase of the G2 population, and this is associated with a decline of BrdU incorporation, arising from the inhibition of S-phase entry by p21. The initial decrease observed in G1 during block formation can be attributed to an overlap of cell populations in the flow histogram. In 4197 cells, addition of the drugs close to the estimated maximum expression of the G2 block shows that pentoxifylline and A802710 effectively reduce the G2 population

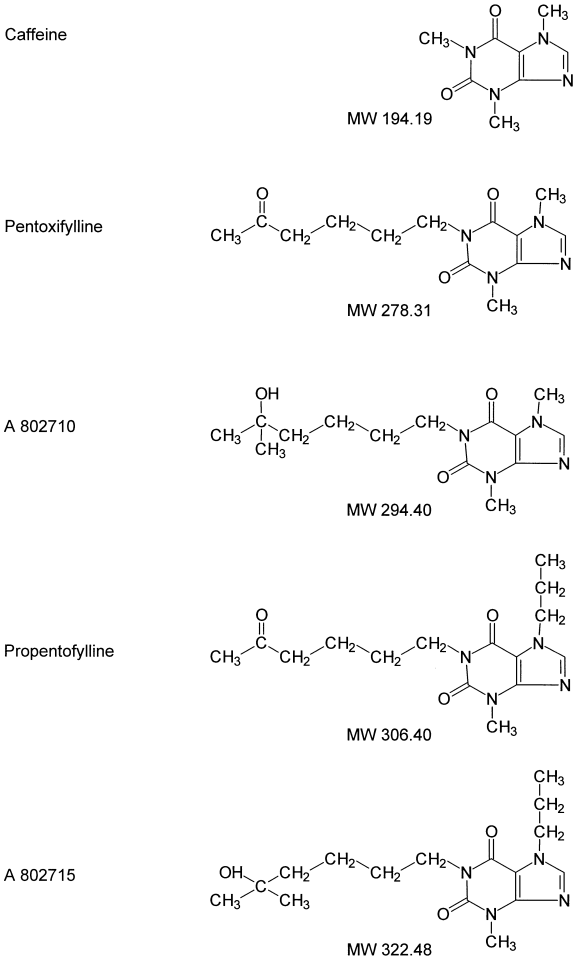

Fig. 1. Methylxanthine derivatives examined were caffeine and the four Hoechst drugs, pentoxifylline, propentofylline, A802715 and A802710.

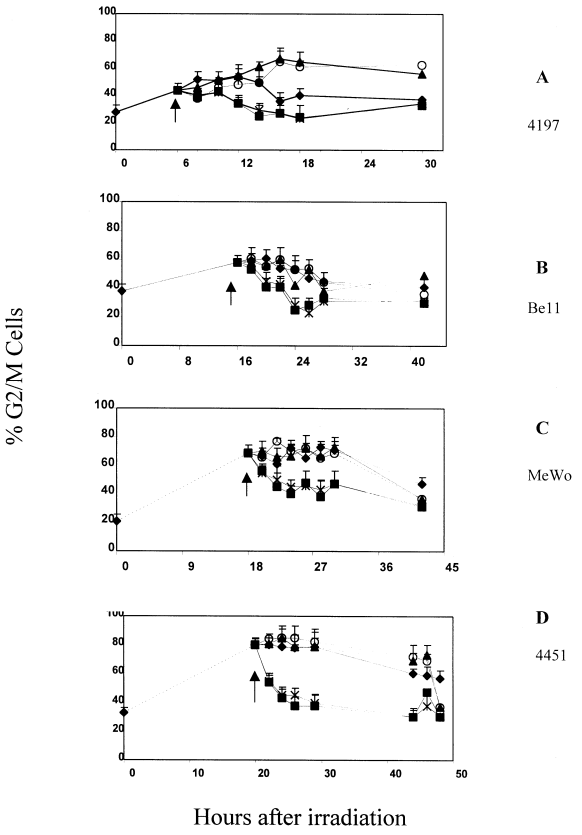

Fig. 2. Influence of methylxanthine derivatives on the irradiation-induced G2/M block in p53 wt 4197 (A) and Be11 (B) cell lines, and in p53 mutant MeWo (C) and 4451 (D) cell lines. The arrow indicates addition of the drug, which was close to the maximum expression of the cell cycle block. Error bars represent 1 S.D. ♦, irradiation only; ■, irradiation+2 mM pentoxifylline; ▲, irradiation+1 mM propentofylline; ○, irradiation+1 mM A802715; *, irradiation+2 mM A802710

below control level, whereas propentofylline and A802715 elevate the G2 population (Fig. 2A). In the Be11 melanoma cell line, propentofylline and A802715 remain ineffective in influencing the G2 population, whereas pentoxifylline and A802710 clearly suppress the G2 population (Fig. 2B). Pentoxifylline and A802710 have no pronounced effect on S-phase contents (Fig. 3A,B) which confirms that the G2 block, but not the G1 block, is abrogated in wt cells. The influence of the other drugs on BrdU incorporation is noticeable in the case of propentofylline and A802715 which strongly enhance the irradiation-induced suppression of S-phase contents (Fig. 3A,B) and this effect is more strongly expressed with A802715.

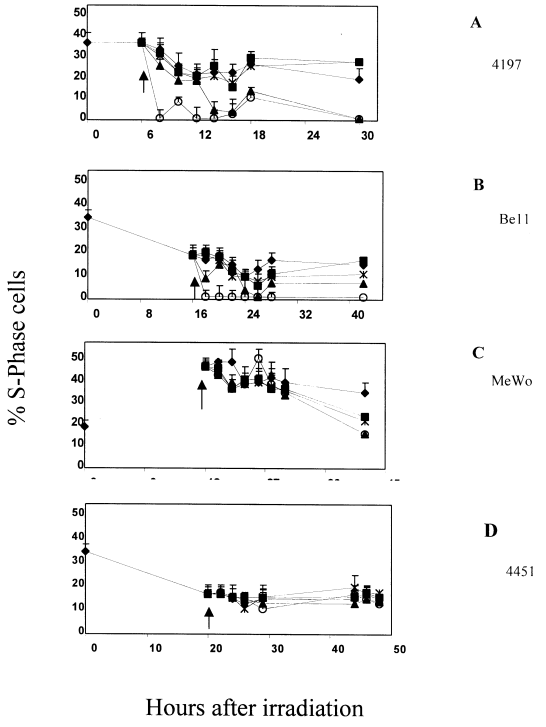

Fig. 3. Influence of methylxanthine derivatives on DNA synthesis in p53 wt 4197 (A) and Be11 (B) cell lines, and in p53 mutant MeWo (C) and 4451 (D) cell lines. Arrow indicates addition of the drug, which was close to the maximum expression of the cell cycle block. S-phases were quantitated by double parameter analysis of total DNA content (red fluorescence) and incorporated BrdU (green fluorescence) using PI and anti-BrdU antibodies labelled with FITC. Error bars represent 1 S.D. ♦, irradiation only; ■, irradiation+2 mM pentoxifylline; ▲, irradiation+1 mM propentofylline; ○, irradiation+1 mM A802715; *, irradiation+2 mM A802710

In p53 mutant cells, irradiation induces a strong decline of the G1 population and elevation of the G2 population giving rise to a G2 block (Fig. 2C,D). The G2 block is associated with a strong increase of BrdU incorporation in MeWo cells, which show an increase in the S-phase content from 20 to 47% (Fig. 3C). This may be due to a block in G2, while the progression of cells from G1 into S-phase continues normally. Interestingly, a similar phenomenon is not observed in the 4451 p53 mutant cell line (Fig. 3D). In p53 mutant cells, the increase of the G2 population after irradiation is effectively reversed by pentoxifylline and A802710 (Fig. 2C,D) but not by propentofylline and by A802715 (Fig. 2C,D). This shows that pentoxifylline and A802710 are effective G2 block abrogators. The effect of A802710 and pentoxifylline on irradiated p53 mutant cells was also monitored in the G1 population, which rapidly responds to the stimulation of mitosis and re-entry into the cell cycle. Addition of pentoxifylline at maximum expression of the G2 block sharply increases the percentage of G1 cells and produces a characteristic overshoot of the G1 population above the level of non-irradiated controls (G1 data not shown). In p53 mutant cells, which exhibit no G1 cell cycle block, the methylxanthine drugs do not influence S-phase as measured by BrdU incorporation (Fig. 3C,D).

When G2 blocks were induced with the spindle inhibitor nocodazole instead of irradiation, the block was unresponsive to pentoxifylline in p53 wt (Fig. 4A,B) and p53 mutant cells (Fig. 4C,D). A 7 h exposure to pentoxifylline completely abrogates the irradiation-induced G2 block (Fig. 5A,B), whereas a 6 h exposure to pentoxifylline in nocodazole-treated cells remains essentially ineffective in reducing the G2 population (Fig. 4B,D). In the nocodazole experiments, exposure to pentoxifylline for up to 10 h was found to be ineffective and G2 populations remained at the level of 40% in p53 wt cells and at 80% in p53 mutant cells. This shows that pentoxifylline does not reverse the inhibition of spindle assembly and operates at another level.

4. Discussion

4.1. Pentoxifylline and A802710

The cytotoxicity of methylxanthine derivatives varies between cell lines. Pentoxifylline and A802710 emerge as the least toxic whereas A802715 and propentofylline are distinctly more toxic. The lower toxicity of pentoxifylline and A802710 renders these drugs more attractive for clinical application than caffeine [3,23]. The effectiveness of pentoxifylline in abrogating the G2 block in p53 mutant cells has been analyzed [1–3,5–8]. Early resumption of the cell cycle progression after DNA damage clearly is an attractive avenue for further intervention with cytotoxic drugs [24].

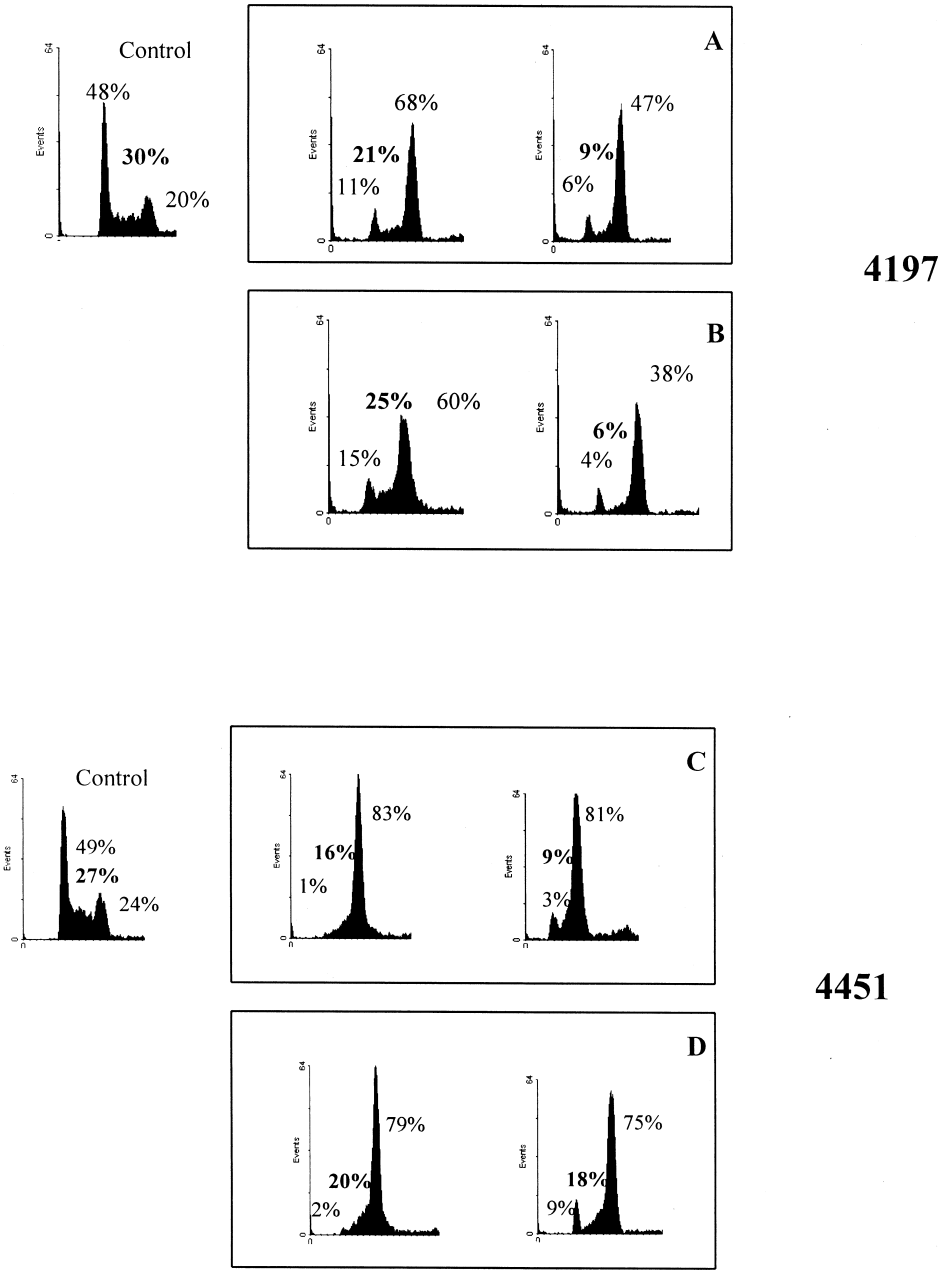

Fig. 4. Influence of pentoxifylline on nocodazole-induced mitotic blocks in p53 wt 4197 cells (A and B) and p53 mutant 4451 cells (C and D). Nocodazole was added to the culture medium at 0.4 μg/ml. When the mitotic block had reached a maximum at 14–16 h in 4197 and 4451 (A and C) cells, 2 mM pentoxifylline was added. Presence of pentoxifylline for 6–10 h did not abrogate the nocodazole-induced mitotic block and the G2/M population remained essentially unchanged (B and D).

4.2. Propentofylline and A802715

These two xanthine derivatives differ markedly from pentoxifylline and A802710 in that they do not inhibit the G2/M block. The G2 versus time relationship for these two drugs is very similar in all four cell lines. A close functional resemblance is particularly apparent in 4197 p53 wt cells where these two drugs prolong the G2/M population by a

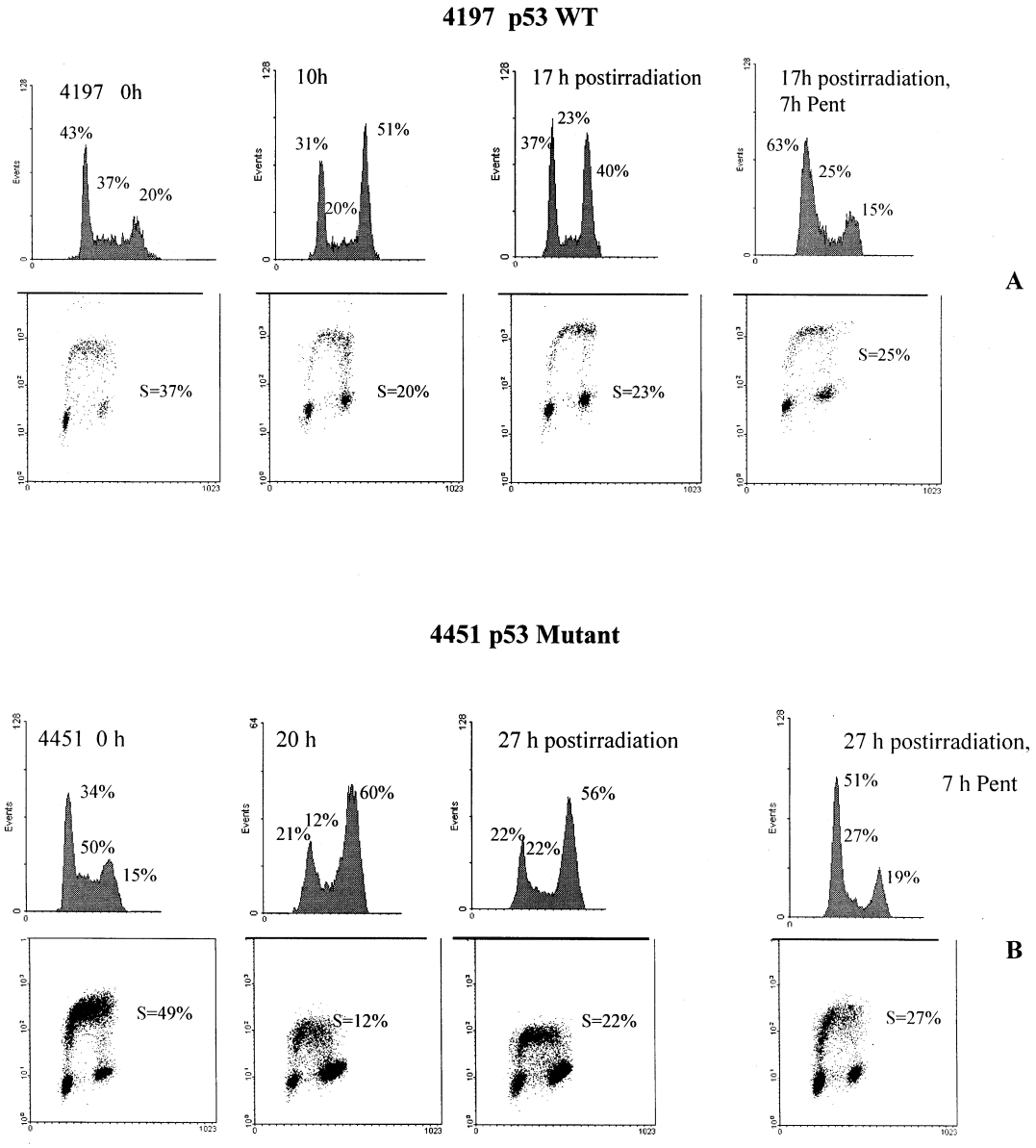

Fig. 5. DNA histograms and dot plots from BrdU incorporation in 4197 p53 wt and 4451 p53 mutant cells in response to cobalt γ-irradiation and addition of pentoxifylline at the G2/M maximum.

similar margin, whereas pentoxifylline A802710 suppresses it (Fig. 2A).

Inspection of S-phase contents in the two p53 wt cells show that propentofylline and A802715 enhance the suppression of BrdU incorporation induced by irradiation (Fig. 3A,B). In the MeWo p53 mutant cell line the S-phase content is elevated after irradiation (Fig. 3C). This is interpreted as an inhibition of S-phase progression [14,25] which is cell type specific, as it is not observed in the 4451 p53 mutant cell line. The continuation of replication in the absence of mitosis (endoreplication) is known to give rise to higher ploidy and such effects were indeed observed in the MeWo cells (data not shown). A802715 and propentofylline suppress entry of irradiated cells into S-phase in p53 wt cells, which can be attributed to an active G1 cell cycle block (Fig. 3A,B). Since all drugs were employed at or near the TD_{50} these results could not arise from toxicity differences or simply from cell kill. The effectiveness of propentofylline

and A802715 in suppressing the entry of cells into S-phase sharply contrasts with pentoxifylline, which has little or no effect on S-phase entry (Fig. 3A,B). Due to the lack of a functional G1 checkpoint, the four drugs have little or no effect on S-phase populations in the two p53 mutant cell lines (Fig. 3C,D). Propentofylline and A802715 prolong the G2 block induced by irradiation, or leave it unaffected, and thus emerge as antagonists to caffeine, pentoxifylline and A802710. Propentofylline and A802715 have been found to exhibit a very strong capacity to suppress tumor necrosis factor α (TNF-α) and inhibit 3′,5′-c-AMP phosphodiesterase (PDE) activity. A802715 has been found to be the most potent TNF-α inhibitor and this inhibitory capacity correlates with the PDE inhibition of the xanthine drug [26,27]. Other studies indicated that A802715 is much more potent than pentoxifylline in the mixed lymphocyte reaction and hence emerge as a strong immune-suppressant [28]. Propentofylline has been shown to inhibit adenosine transport into cells and increases extra-cellular adenosine concentrations thereby simulating adenosine receptors and exhibiting a neuroprotective effect far in excess of pentoxifylline [29].

The remarkable functional similarity between propentofylline and A802715 raises the question of structural interrelationships. Caffeine, pentoxifylline and A802710 carry a methyl substituent at N7 (Fig. 1) whereas propentofylline and A802715 carry a propyl residue at N7. Since G2 block inhibition is shown by caffeine [8,30,33–37], pentoxifylline and A802710, which are N7-methyl, it appears that the effectiveness of these three drugs as G2/M block abrogators may lie in the N7 substituent. Variations in N1 substituent (methyl for caffeine, 5-oxohexyl for pentoxifylline and 5-hydroxyhexyl for A802710) are of little consequence for the G2 block abrogator activity, but replacement of the N7 methyl group by a propyl group in A802715 and in propentofylline abolishes the G2 block inhibition activity. This suggests that the activity of methylxanthines in cell regulation may indeed rest in the N7 substituent. The use of other model compounds would help to corroborate this impression.

Methylxanthines are potent PDE inhibitors [38,39]. It is also well known that the association of cyclin B1 and cyclin-dependent kinases into the active cyclin B1/p34^{cdc2} complex is regulated by activating phosphatases [40]. The possibility, therefore, exists that pentoxifylline inhibits this activation step. Alternatively, the drug could influence the cyclin B1 constituent. The use of anti-cyclin B1 antibodies indeed have shown that irradiation decreases cyclin B1 expression and that pentoxifylline restores the cyclin B1/G2 ratio to control levels [41]. Elevation of cyclin B1 expression, therefore, must be considered as part of the mechanism of action of pentoxifylline as a G2 block abrogator. The fact that pentoxifylline does not abrogate G2 blocks induced by the microtubule inhibitor nocodazole (Fig. 4), suggests that pentoxifylline does not operate at the spindle assembly checkpoint.

Our studies on the influence of methylxanthine derivatives on cell regulation demonstrate that only the two N-7 methyl derivatives effectively abrogate the damage-induced G2 cell cycle block, whereas the two N-7 propyl derivatives are ineffective. The effectiveness of N-7 methyl in G2 block abrogation also applies to caffeine [41]. The N-7 propyl derivatives also differ from the N-7 methyl derivatives in that they enhance the suppression of the S-phase after irradiation in p53 wt cells, but show no effect on the S-phase in irradiated p53 mutant cells. The functional distinction of the two groups of drugs in terms of cell regulation suggests a differential influence on cyclin-dependent kinases and a mechanism involving the activation and intracellular location of the cyclin B1/p34^{cdc2} complex has been considered [42–44].

Acknowledgements

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